Biochemical Pharmacology, Vol. 32, No. 8, pp. 1454–1456, 1983.
Printed in Great Britain.

0006-2952/83/081454-03 $03.00/0
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Inhibition of mitochondrial nucleic acid synthesis by methyl mercury

(*Received* 17 *September* 1982; *accepted* 7 *December* 1982)

Methyl mercury (MeHg) has been known for over a decade to be an environmentally important toxic chemical [1–4]. Over the past few years, a number of studies have demonstrated the toxicity of the chemical at the cellular level [5]; however, the mechanism of cellular toxicity of MeHg remains unknown.

There have been several studies on the effects of MeHg on cellular macromolecular synthesis [5–11]. Studies on nucleic acid synthesis have examined its effects on total DNA and RNA synthesis in the cell, which, of course, is primarily nuclear. However, mitochondria possess their own DNA, which undergoes replication and transcription [12], and recent reports have suggested that effects on mitochondria may be an important component of mercury-induced toxicity [13, 14]. Furthermore, recent findings have suggested that mitochondrial DNA may be a major and perhaps even preferential target for some chemical carcinogens [15–17]. We therefore felt that an examination of the effects of MeHg on nucleic acid synthesis in mitochondria would be important for obtaining a complete picture of the toxic effects of the compound. In addition, in a recent study of nucleic acid synthesis *in vitro* in isolated nuclei, we found that MeHg specifically stimulated RNA synthesis catalyzed by RNA polymerase II but inhibited RNA synthesis catalyzed by polymerases I and III, as well as DNA synthesis [18]. Since isolated mitochondria also carry out synthesis of DNA and RNA *in vitro* (reviewed in Refs. 19 and 20), it was also of interest to compare the effects of MeHg on nucleic acid synthesis in isolated mitochondria with its effects on synthesis in isolated nuclei.

Materials and Methods

Cells. HeLa cells were grown in suspension cultures in Joklik's modified minimal essential medium (GIBCO, Grand Island, NY) containing 5% horse serum.

Materials. Unlabeled nucleoside triphosphates were purchased from P.L. Biochemicals (Milwaukee, WI) and radioactive nucleoside triphosphates from the New England Nuclear Corp. (Boston, MA). Methyl mercury hydroxide was purchased from the Alfa Chemical Co. (Danvers, MA), ethidium bromide from Calbiochem (La Jolla, CA), and α-amanitin from the Sigma Chemical Co. (St. Louis, MO).

Nucleic acid synthesis in whole cells. Cells were suspended at a density of 2×10^6/ml (total 10 ml) and incubated at 37° for 2 hr with [^{3}H]thymidine (5 μCi/ml; 76 Ci/mmole) or [^{3}H]uridine (5 μCi/ml; 38 Ci/mmole) together with the indicated concentration of MeHg. Blanks were obtained by keeping the cultures at 0° for the 2-hr period. After the incubation, the cells were washed twice with 0.15 M NaCl containing 5 mM KCl and 1 mM $MgCl_2$, once with 10 mM Tris–HCl buffer (pH 8.1) containing 0.1 mM EDTA and 0.25 M sucrose, and resuspended in the same buffer at a density of 5×10^6 cells/ml. After dounce homogenization, the nuclei were collected by centrifugation for 4 min at 1500 *g*, washed once with the same buffer, and resuspended in 1 ml of 10 mM EDTA containing 0.1% sodium dodecyl sulfate (final pH = 7.4) to which 1 ml of 10% trichloroacetic acid was added. The precipitate was collected on a Whatman GF/C filter, washed three times with cold 0.01 N HCl, and dried. The radioactivity was determined in a liquid scintillation spectrometer.

The supernatant fraction from the centrifugation in which the nuclei were pelleted was centrifuged for 10 min at 10,000 *g*. After repeated centrifugation to remove residual nuclei, the mitochondrial pellet was resuspended in 10 mM Tris–HCl buffer (pH 8.1) containing 0.1 mM EDTA and 0.25 M sucrose, centrifuged again, and resuspended in 1 ml of 10 mM EDTA containing 0.1% sodium dodecyl sulfate (final pH = 7.4) to which 1 ml of 10% trichloroacetic acid was added. The precipitate was collected, and the radioactivity was determined as for the nuclear fraction.

To determine cytoplasmic acid-precipitable radioactivity, 1 ml of 10% trichloroacetic acid was added to a 1-ml aliquot of the supernatant fraction of the centrifugation in which the mitochondria had been pelleted. The precipitate was collected, and the radioactivity was determined as for the nuclear fraction.

Isolation of mitochondria and nuclei for in vitro *synthesis.* Nuclei were isolated from dounce-homogenized cells as described previously [18]. After centrifugation of the cell lysate to remove the nuclei (10 min at 1500 *g*), the supernatant fraction was centrifuged repeatedly until no residual nuclear pellet was seen. The supernatant fraction was then centrifuged for 10 min at 10,000 *g* to pellet the mitochondria, which were then washed once with 50 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes) buffer (pH 7.4) containing 100 mM NaCl and 1 mM $MgCl_2$ and resuspended in the same buffer (at a concentration of 8×10^8 cell equivalents/ml for DNA synthesis or 2.5×10^9 cell equivalents/ml for RNA synthesis).

Synthetic reactions were carried out with 0.1 ml of isolated nuclei or mitochondria, using the reaction mixture appropriate for DNA or RNA synthesis, as described previously [18].

Results and Discussion

It has been shown by Gruenwedel and Cruikshank [7] and confirmed in this laboratory [18] that DNA and RNA synthesis in intact cells is inhibited by MeHg. To distinguish the two basic subcellular types of nucleic acid synthesis, i.e. nuclear and mitochondrial, cells which had been exposed to MeHg and [^{3}H]thymidine or [^{3}H]uridine were fractionated into nuclear, cytoplasmic, and mitochondrial fractions, and the acid precipitable radioactivity in each fraction was determined. As expected, nuclear DNA and RNA synthesis, which represents >95% of the total cellular synthesis, was inhibited by MeHg (Fig. 1). The effect of MeHg on mitochondrial nucleic acid synthesis was virtually identical to its effect on nuclear synthesis.

To be certain that the synthesis measured in this experiment was in fact mitochondrial, the effect of ethidium bromide on the synthesis was examined. This compound has been shown to selectively inhibit mitochondrial nucleic synthesis *in vivo* and *in vitro* [19–23]. The results showed that incorporation of [^{3}H]thymidine or [^{3}H]uridine into the mitochondrial fraction was inhibited 50% by ethidium bromide (at a concentration of 1 μg/ml), whereas incorporation into the nuclei-plus-cytoplasm fraction was either unaffected or stimulated.

In an earlier study [18], we examined the effects of MeHg on nucleic acid synthesis in isolated nuclei *in vitro* and observed a specific stimulation of synthesis which was catalyzed by RNA polymerase II. To further examine the specificity of this phenomenon, it was of interest to examine

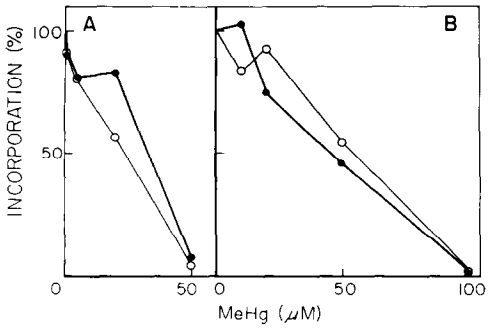

Fig. 1. Effect of MeHg on nucleic acid synthesis in whole cells. Incorporation of [^{3}H]thymidine or [^{3}H]uridine into DNA or RNA, respectively, in the various subcellular functions was measured in the presence of the indicated concentration of MeHg, as described in Materials and Methods. (A) DNA: (○) nuclei-plus-cytoplasm (100% = 6.3×10^5 cpm); and (●) mitochondria (100% = 1.6×10^4 cpm); (B) RNA: (○) nuclei-plus-cytoplasm (100% = 5.2×10^5 cpm); and (●) mitochondria (100% = 2.4×10^4 cpm).

To determine the specificity of the synthesis by our preparation of isolated mitochondria, ethidium bromide was again employed. As shown in Fig. 2, synthesis of DNA and RNA *in vitro* by a mitochondrial preparation was inhibited by ethidium bromide, whereas synthesis by nuclei under the same conditions was unaffected. The bulk of the synthesis *in vitro* by the preparation was thus clearly mitochondrial. The effect of MeHg on DNA synthesis by isolated nuclei and mitochondria is illustrated in Fig. 3. The results show that mitochondrial DNA synthesis was inhibited by MeHg; the sensitivity of DNA synthesis in the two organelles was similar, with mitochondria exhibiting perhaps slightly less sensitivity. *A priori*, preferential inhibition of mitochondrial DNA synthesis might have been anticipated because of the presence of single-stranded regions in replicating mitochondrial DNA [24] and the binding of MeHg to single stranded DNA [25]. Clearly, however, no such preferential inhibition occurred, either *in vivo* or *in vitro* (Figs. 1A and 3).

The differential effects of MeHg on nuclear α-amanitin-sensitive and -resistant RNA synthesis *in vitro*

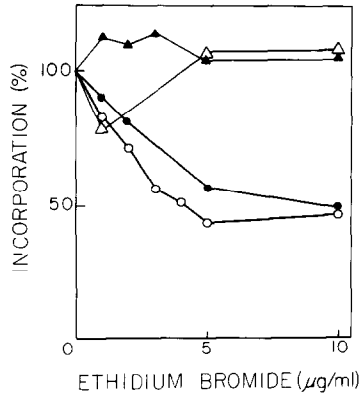

Fig. 2. Effect of ethidium bromide on nucleic acid synthesis in isolated mitochondria and nuclei. Mitochondria and nuclei were isolated, and nucleic acid synthesis was measured as described in Materials and Methods. Key: (○) DNA synthesis, mitochondria (100% = 1.3×10^3 cpm); (△) DNA synthesis, nuclei (100% = 2.9×10^3 cpm); (●) RNA synthesis, mitochondria (100% = 1.3×10^3 cpm); and (▲) RNA synthesis, nuclei (100% = 1.8×10^4 cpm).

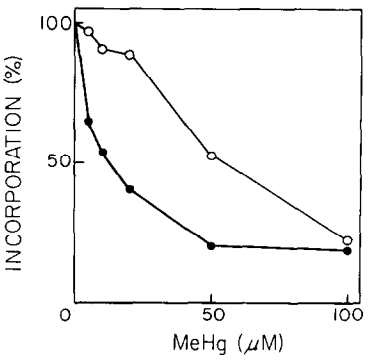

Fig. 3. Effect of MeHg on DNA synthesis in isolated mitochondria and nuclei. Experimental conditions were described in Materials and Methods. Key: (○) mitochondria (100% = 2.7×10^3 cpm); and (●) nuclei (100% = 1.3×10^3 cpm).

are illustrated in Fig. 4. This figure also shows that MeHg inhibited mitochondrial RNA synthesis; the dose–response appears similar to that of nuclear α-amanitin-resistant synthesis. Although mitochondrial RNA polymerases have not yet been unambiguously characterized, it seems clear that mitochondria do possess a distinct enzyme which resembles nuclear RNA polymerase I in its insensitivity to α-amanitin [26, 27]. These results indicate that the enzymes are also similar in their sensitivities to MeHg and further indicate that the stimulatory effect of MeHg on RNA synthesis *in vitro* is specific for RNA polymerase II-catalyzed synthesis in isolated nuclei.

In summary, the effects of methyl mercury on mitochondrial synthesis of DNA and RNA in HeLa cells have been investigated and compared to its effects on nuclear synthesis. In intact cells the compound inhibited both DNA and RNA synthesis in mitochondria at doses which were virtually identical to those which inhibited nuclear synthesis. DNA synthesis in isolated mitochondria was also inhibited by methyl mercury, but the synthesis was somewhat less sensitive than nuclear DNA synthesis. The effect

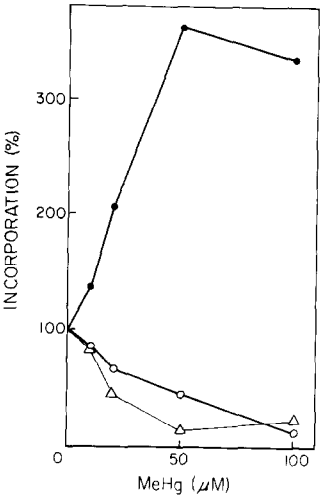

Fig. 4. Effect of MeHg on RNA synthesis in isolated mitochondria and nuclei. Experimental conditions were as described in Materials and Methods. Key: (○) nuclei, α-amanitin (0.5 μg/ml) resistant synthesis (100% = 2.6×10^3 cpm); (●) nuclei, α-amanitin-sensitive synthesis (total minus resistant) (100% = 1.1×10^3 cpm); and (△) mitochondria (100% = 1.6×10^3 cpm).

of methyl mercury on RNA synthesis in isolated mitochondria was similar to its effect on α-amanitin-resistant RNA synthesis in isolated nuclei, i.e. inhibition (in contrast to α-amanitin-sensitive RNA synthesis in isolated nuclei which was stimulated by methyl mercury).

Acknowledgements—This work was supported in part by Grant ES-02642 from the National Institute of Environmental Health Sciences, and Grant 11-010 from the New York State Health Research Council.

Center for Laboratories and Research
New York State Department of Health
Albany, NY 12201, U.S.A.

GERALD D. FRENKEL*
LISA HARRINGTON

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* Author to whom correspondence should be addressed.